purple in air within a few days, while, under nitrogen, it remained almost colorless for prolonged periods (*cf.* ref. 26).

Attempted recrystallization from organic solvents or aqueous solvent mixtures resulted in rapid and extensive decomposition with the formation of intensely red or purple products. However, recrystallization could be effected satisfactorily from aqueous methanol containing a little sodium bisulfite. Several recrystallizations in this fashion afforded a sample for analysis as colorless, glistening leaflets, dec. *ca.* 100°; λ_{max}(Nujol) 2.95, 3.70(br), 6.25(br) μ.

Anal. Calcd. for $C_9H_9N_3O$: C, 61.70; H, 5.18; N, 23.99. Found: C, 61.70; H, 5.19; N, 23.89.

4-Diazo-3-phenyl-5-pyrazolone (XXXIII).—Compound XXXII (1.75 g., 0.010 mole) was dissolved in a mixture of ice and concentrated sulfuric acid, and a solution of sodium nitrite (1.0 g., 0.014 mole) in a little water was added slowly to the stirred mixture. The deep yellow slurry was poured into dilute aqueous sodium bicarbonate. The deep yellow, crystalline deposit upon recrystallization from benzene afforded yellow-orange, glistening leaflets (1.3 g., 70%), m.p. 180–181° dec. Several recrystallizations from benzene afforded a sample, m.p. 182–183°, for analysis; λ_{max}(CH_2Cl_2) 2.95, 4.78, 5.93 μ.

Anal. Calcd. for $C_9H_6N_4O$: C, 58.06, H, 3.25; N, 30.10. Found: C, 58.29; H, 3.41; N, 30.04.

This product dissolved in dilute aqueous sodium hydroxide to give yellow solutions from which it was reprecipitated upon acidification.

Sodium Hydrosulfite Reduction of XXXIII. Formation of 4-Amino-3-phenyl-5-pyrazolone (XXXII).—Compound XXXIII (190 mg., 1.0 mmole) was dissolved in boiling 80% aqueous ethanol, and powdered sodium hydrosulfite was added in small portions. Immediate, vigorous gas evolution was evident, and the mixture turned red. Deep yellow-orange leaflets slowly deposited, then went back into solution with the further addition of sodium hydrosulfite to the boiling mixture. A colorless solution eventually resulted. The solution was concentrated to a small volume, saturated with ammonium chloride, cooled and scratched. The colorless crystals which deposited (100 mg., 57%), dec. *ca.* 100°, had an infrared spectrum identical with that of 4-amino-3-phenyl-5-pyrazolone (XXXII).

Attempted Decomposition of XXXIII in Methanolic Sodium Hydroxide.—Compound XXXIII (190 mg., 1.0 mmole) was dissolved in warm methanol (*ca.* 5 ml.) and the yellow-orange solution was treated with several drops of aqueous 40% potassium hydroxide. No gas evolution was evident. The solution was boiled under reflux for 30 minutes, neutralized with acetic acid, diluted to the cloud point with water and chilled to −10°. The yellow-orange needles thus formed (180 mg., 95%), m.p. 180–181°, were shown to be identical with the starting material by infrared spectral comparison and a mixture melting point determination.

Attempted Thermal Decomposition of XXXIII.—Compound XXXIII (0.50 g., 2.7 mmoles) was dissolved in hot xylene (*ca.* 20 ml.), and the yellow-orange solution was boiled under reflux for 12 hours. Upon slow cooling to 5°, deep yellow-orange, glistening leaflets deposited. This substance (0.45 g., 90%), m.p. 180–181° dec., was shown to be identical with the starting material by infrared spectral comparison and a mixture melting point determination.

Pyrolysis of XXXIII in Benzyl Alcohol. Formation of 4-Benzyl-3-phenylpyrazolone (XXXIV).—Compound XXXIII (380 mg., 2.0 mmoles) was dissolved in hot benzyl alcohol (*ca.* 25 ml.) and the deep yellow-orange solution was gradually brought to the boiling point. Slow gas evolution began at *ca.* 160°. The solution was boiled under reflux for 4 hours, during which time it turned deep red, then finally became pale yellow. The reaction mixture was poured into water, and benzyl alcohol was steam distilled from the mixture. The mixture was cooled and the light yellow solid was extracted with ethyl acetate. The organic extracts were dried over sodium sulfate and evaporated to dryness on the steam-bath; the residue was crystallized from benzene. The colorless crystals thus obtained (180 mg., 51%), m.p. 179.5–181.5°, upon recrystallization from benzene afforded an analytical sample, m.p. 185–186°; λ_{max} (Nujol) 2.98, 3.9 (br), 6.65 μ.

Anal. Calcd. for $C_{16}H_{14}N_2O$: C, 76.78; H, 5.64; N, 11.19. Found: C, 77.04; H, 5.29; N, 10.92, 11.67.

The substance was soluble in dilute aqueous sodium hydroxide to give a colorless solution from which it was recovered unchanged upon acidification, and gave an intense red color with ferric chloride in ethanol.

4-Benzyl-3-phenyl-5-pyrazolone (XXXIV).—3-Phenyl-5-pyrazolone[36] (1.6 g., 10 mmoles) was dissolved in hot benzyl alcohol, and benzaldehyde (5 drops) was added. The resultant solution was boiled under reflux for 4 hours, cooled and poured into water. Benzyl alcohol was then steam distilled from the resultant mixture. Upon cooling, the remaining insoluble oil solidified and was collected. Recrystallization from benzene, after filtration of the hot, benzene solution from some insoluble material, afforded colorless needles of XXXIV (1.0 g., 40%), m.p. 184–185° (lit.[28] 186°). The melting point of this substance was undepressed on admixture with the product obtained from pyrolysis of XXXIII in benzyl alcohol. The infrared spectra of these compounds were identical.

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Cytidine 5′-Sulfate and Related Nucleotide Analogs

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Synthesis of pentose-sulfated analogs of cytidine ribonucleotides has been achieved by 3 methods: (i) reaction of chlorosulfonic acid with cytidine, yielding 7 different cytidine sulfates, (ii) reaction of chlorosulfonic acid with 2′,3′-*O*-isopropylidene followed by mild acid hydrolysis, yielding cytidine 5′-sulfate only and (iii) reaction of sulfuric acid with cytidine at 60°, yielding the same cytidine derivatives as Method i. Procedures for separation of products are presented. Paper chromatographic, electrophoretic, ion exchange, spectrophotometric, radiochemical and acid degradation studies were applied to characterize products and investigate their properties. All evidence indicates that the products are cytidine 2′-sulfate, cytidine 3′-sulfate, cytidine 5′-sulfate, cytidine 2′,3′-disulfate, cytidine 2′,5′-disulfate, cytidine 3′,5′-disulfate and cytidine 2′,3′,5′-trisulfate.

Egami and Takahashi[2] have synthesized adenosine sulfates (adenosine sulfuric acids) by reaction of chlorosulfonic acid with adenosine in pyridine solution. Ion exchange chromatography resolved the product mixture into three fractions characterized as adenosine mono-, di- and tri-sulfates. The adenosine monosulfate product competes with the natural nucleotides, diphosphopyridine nucleotide,[3] flavin adenine dinucleotide[4] and adenosine 5′-monophosphate[5] in certain of their enzyme-

(1) In partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

(2) F. Egami and N. Takahashi, *Bull. Chem. Soc. Japan*, **28**, 666 1955).

(3) I. Yamashina and F. Egami, *J. Japan Biochem. Soc.*, **25**, 281 (1953).

(4) F. Egami and K. Yagi, *J. Biochem. Japan*, **43**, 153 (1956).

(5) M. Niwa, S. Higuchi and F. Egami, *ibid.*, **45**, 89 (1958).

catalyzed reactions. Such metabolic antagonism to purine nucleotides suggests that sulfate analogs of natural pyrimidine nucleotides may also prove to be of interest. In the present report, synthesis, separation, identification and some properties of cytidine sulfates are described with emphasis on cytidine 5′-monosulfate (1-β-D-ribofuranosyl-2-oxo-4-amino-pyrimidine 5′-sulfate) (5′-CMS).[6]

Experimental

Fractionating Systems.—Paper chromatographic separation of the products of synthesis was done in Whatman 3 MM paper with solvent 1, isobutyric acid: 0.5 *N* ammonium hydroxide (5:3),[7] and solvent 2, saturated aq. ammonium sulfate: water: 2-propanol (79:19:2).[8] Other solvents used were solvent 2a, saturated aq. ammonium sulfate: 0.5 *M* pH 7 phosphate buffer: 2-propanol (79:19:2), solvent 3, *n*-propanol: 14.8 *N* ammonium hydroxide: water (6:3:1)[9] and solvent 4, 2-propanol: glacial acetic acid: water (6:3:1).[10] Overnight shaking in water saturated with chloroform was used to elute the compounds from paper. Adsorption of cytidine derivatives on Norite A and desorption with ethanol: 1 *N* ammonium hydroxide[11] served to remove ammonium sulfate present in eluates of compounds from chromatograms developed in solvent 2. Paper electrophoresis was done in Whatman 3 MM paper using a water-cooled apparatus with 31 cm. paper length between electrolyte surfaces. Anion exchange fractionations were done with Keir and Davidson's modification of a formic acid gradient eluting system[12] using Dowex-1 resin.

Assay of Products.—For routine analysis, the OD_{max} of cytidine and the cytidine sulfates in 0.1 *N* HCl was measured against an appropriate blank with a calibrated Beckman DU spectrophotometer. The ϵ_{max} of 5′-CMS was found to be 13,100. This value was used in the calculations of the concentration of all products and mixtures of them.

Syntheses and Fractionation of Products.—In the course of the investigations 3 synthetic methods[13] were developed for different purposes: I was designed for relatively large scale preparation of cytidine sulfates, II for specific synthesis of 5′-CMS and III primarily for synthesis of S^{35}-labeled cytidine sulfates on micro or ultramicro levels.

Method I.—Cytidine hemisulfate (0.99 mmole containing 1.98 mmoles cytidine) from Schwarz Biochemical Corp. was dissolved in 40 ml. of dry pyridine. Chlorosulfonic acid (0.4 ml.) was added at 0°. The reaction mixture was stirred overnight at 2° and allowed to stand 1 hr. at room temperature. Most of the pyridine was removed by decantation and the remaining viscous syrup diluted to 8.0 ml. with water. TOD_{280} = 23,400 as determined on an aliquot freed of pyridine by two evaporations from 0.1 *N* lithium hydroxide.

Part of the crude product (0.7 ml.) was fractionated on an analytical scale by two dimensional chromatography in solvents 1 and 2 (approx. 3 mg. total cytidine and derivatives per chromatogram). As shown in Fig. 1, the development in solvent 1 yielded 5 product deposits, the second and fifth of which were resolvable into 2 components by development with solvent 2 in the second dimension. Two other deposits recognizable as unreacted cytidine and pyridine were situated +Y of the area of the chromatogram that has been included in Fig. 1. No additional ultraviolet-absorbing compounds could be detected by using other solvents or electrophoresis instead of solvent 2 for development in the second dimension. Preliminary identification of the products as shown in Fig. 1 was made by analogy of paper chromatographic characteristics to those of corresponding cytidine phosphates.[15]

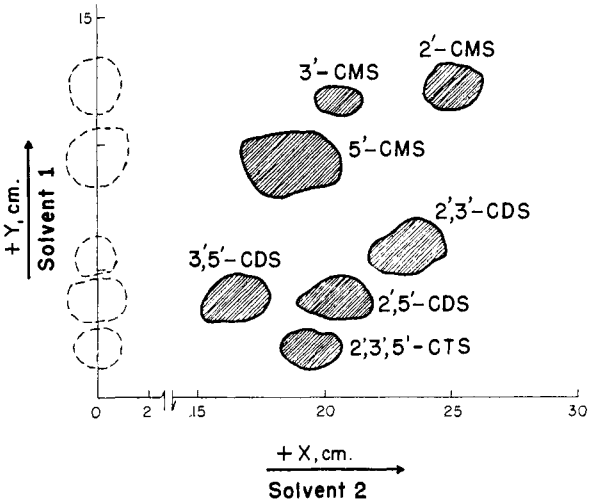

Fig. 1.—Distribution of the cytidine sulfates from synthesis by Method I in a typical two-dimensional paper chromatogram. Dashed lines delineate location of deposits following development 1 only; solvent 1, 24 hr. descending flow +Y direction. Shaded areas show distribution of compounds after development 2; solvent 2, 18 hr. descending +X.

Cytidine 5′-sulfate was isolated by preparative scale paper chromatography of the crude product. 7.0 ml. of the above solution was applied linearly to 6 chromatographic papers, 0.032 ml. per cm. 100 μg. of 5′-CMP was applied near the end of each applied band as a mobility reference compound. Papergrams were developed in solvent 1 with ascending flow for 20 hr., then with descending flow for 20 hr. in the same solvent. Five product bands corresponding to the five spots detected on the analytical chromatograms (dashed outlines of Fig. 1) were outlined with aid of an ultraviolet fluoroscope.[11] In each chromatogram the very strong band having an $R_m \cong 0.82$ was eluted with water. The eluate from paper was fractionated by anion exchange chromatography to remove ammonium ion. Anion exchange eluate fractions of optical density > 0.1 had a TOD_{280}

(6) Abbreviations as follows: CMS, CDS and CTS = cytidine mono-, di- and tri-sulfates, respectively. CMP and CDP = cytidine mono- and di-phosphates, respectively. Numbers 2′, 3′ and/or 5′ preceding these abbreviations designate position(s) of attachment of sulfate or phosphate to ribose moiety. *TOD* = optical density measured with 1 cm. light path × volume in ml. R_m = mobility of a compound in a fractionating system relative to mobility of 5′-CMP. M.R. = molar radioactivity.

(7) B. Magasanik, E. Vischer, R. Doniger, D. Elson and E. Chargaff, *J. Biol. Chem.*, **186**, 37 (1950).

(8) R. Markham and J. D. Smith, *Biochem. J.*, **49**, 401 (1951).

(9) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(10) J. Montreuil and P. Boulanger, *Compt. rend.*, **231**, 247 (1950).

(11) K. K. Tsuboi and T. D. Price, *Arch. Biochem. Biophys.*, **81**, 223 (1959).

(12) (a) H. M. Keir and J. M. Davidson, *ibid.*, **77**, 68 (1958); (b) H. Busch, R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.*, **196**, 717 (1952); (c) W. E. Cohn, *J. Am. Chem. Soc.*, **72**, 1471 (1950).

(13) Method I is an adaptation of the procedure used by Egami and Takahashi[2] for synthesis of adenosine sulfates, Method II is a composite of standard procedures and Method III was suggested by an observation that the Hurwitz[14] synthesis of cytidine monophosphates yields cytidine monosulfate by-products when the source of cytidine is its hemisulfate salt.

(14) J. Hurwitz, *J. Biol. Chem.*, **234**, 2351 (1959).

(15) The preliminary chromatographic identification of substances of the deposits (Fig. 1) has been supported by all subsequent studies. Cytidine phosphates available for direct comparison were 2′-CMP; 3′-CMP, 5′-CMP, 3′,5′-CDP, and the mixed Ba salts of 2′,5′-CDP and 3′,5′-CDP. The synthetic diphosphates were kindly donated by Dr. C. A. Dekker. Relative mobilities of the five cytidine sulfates (Fig. 1 and Table IV) that are analogs of these available cytidine phosphates invariably have corresponding sequences of R_m values in solvents 1, 3 and 4. In solvent 2 the cytidine phosphates all have very high R_f values and R_m values close to 1.00. However, it could be established that 2′-CMP is completely separable from, and ahead of 3′-CMP on long development with solvent 2 by ascending technique, in agreement with findings on the easily resolved 2′- and 3′-isomers of purine nucleotides[8] and the present results on CMS isomers. Identity of the substance of the CDS deposits was inferred from extrapolation of effects of location of negative charge on mobilities of CMS isomers to effects on CDS isomers. 2′,3′,5′-CTS was tentatively recognized by its low mobility in the relatively non-polar solvents 1, 3 and 4.

= 4,800 and *p*H 1.8 to 2.0.[16] They were pooled and evaporated *in vacuo* to a yellow, semi-crystalline solid. Two recrystallizations from 80% ethanol yielded a white, finely crystalline precipitate. It was washed with ethanol and dried over P_2O_5 at 75° for 3 hr. (0.34 mmole cytidine 5′-sulfate; tetragonal crystals: m.p. 212–215° (decomp.); $\lambda_{max}^{pH\,1}$ 280 mμ (ε 13,100); $\lambda_{max}^{pH\,7}$ 271 mμ (ε 8,900)).

Anal. Calcd. for $C_9H_{13}O_8N_3S$: C, 33.41; H, 4.05; N, 12.99; S, 9.92. Found: C, 33.69; H, 4.15; N, 13.17; S, 9.77.

Cytidine 2′-sulfate and cytidine 3′-sulfate in the preparative chromatograms were resolved by elution of the fifth product band at $R_m \cong 0.96$ and rechromatography in new chromatograms developed with solvent 2. The resulting ultraviolet-absorbing bands at R_m = 0.84 and 0.68 were tentatively designated 2′-CMS and 3′-CMS respectively,[15] eluted and freed of ammonium sulfate by adsorption on Norite. The eluates from Norite were evaporated to dryness *in vacuo* leaving the compounds as semicrystalline ammonium salts.

Substances of deposits designated 2′,3′-CDS, 2′,5′-CDS, 3′,5′-CDS and 2′,3′,5′-CTS (Fig. 1) were eluted from ten of the analytical scale chromatograms. Corresponding eluates were pooled, freed of ammonium sulfate with Norite and saved for the investigations as their dry ammonium salts.

Method II.—2′,3′-*O*-Isopropylidene cytidine was prepared from 2.57 mmoles of cytidine by the method of Chambers, Shapiro and Kurkov.[17] It was dissolved in dry pyridine (75 ml.) and treated with chlorosulfonic acid in chloroform (10 ml. of 1:2 solution) at 0°. The reaction flask was stoppered and stirred for 48 hr. at room temperature. A precipitate adhering to the flask was separated by decantation and augmented by further precipitate from centrifugation of the turbid supernatant solution. Analysis as in Method I revealed that 49% of the TOD_{280} was in the final supernatant solution and 51% was in the precipitate. Paper chromatography of components in solvent 2a revealed a single ultraviolet-absorbing product with R_m = 0.38 (values for cytidine, isopropylidene cytidine and 5′-CMS were 0.80, 0.41 and 0.64, respectively). Paper electrophoresis in borate buffer at *p*H 9.0 showed the product to have an R_m = 0.49 (R_m values of cytidine, isopropylidene cytidine and 5′-CMS being 0.46, 0.00 and 0.91, respectively). The R_m value approx. equal to that of the borate complex of cytidine and one-half that of 5′-CMS attested to identity of the intermediate product as the expected 2′,3′-*O*-isopropylidene cytidine 5′-sulfate, because this compound bears one negative charge on the sulfate group and lacks the proximal hydroxyl groups needed to form a borate complex with negative charge. The analyzed supernatant and precipitate fractions were pooled, treated with sodium hydroxide pellets (150 mmoles) and taken to dryness *in vacuo*. The orange residue was dissolved in water and fractionated by anion exchange chromatography. The intermediate product appeared in fractions that had *p*H 1.7 when pooled. The pooled solution was heated at 100° for 10 min., yielding a trace of cytidine and a major product assumed to be cytidine 5′-sulfate. Solvent was removed *in vacuo*, and the product precipitated and recrystallized as in Method I. Yield = 0.465 mmole.

Method III.—Cytidine (0.34 mmole) and sulfuric acid (0.44 mmole)[18] in 1.6 ml. of aqueous volume were evaporated to dryness in a test tube and heated at 60° for 3 hr. over P_2O_5. Water (2.0 ml.) was added and the sample re-evaporated and heated at 60° for an additional 3 hr. The sample was dissolved in 2.0 ml. of water. Aliquots analyzed by analytical scale chromatography showed seven spots resembling those obtained from the syrup in Method I. 5′-CMS was separated from the remaining solution directly by preparative scale paper chromatography as in Method I. Yield of 5′-CMS based on spectrophotometric measurement of the eluate = 0.11 mmole.

(16) On co-chromatography of 2′-CMS and 3′-CMS with 5′-CMS in the formic acid gradient ion exchange system,[12] the 3 CMS isomers were eluted together at *p*H 1.9. When 5′-CMP was present, it was eluted earlier at *p*H 2.3.

(17) R. W. Chambers, P. Shapiro and V. Kurkov, *J. Am. Chem. Soc.*, **82**, 971 (1960).

(18) To prepare S^{35}-labeled cytidine sulfates, a chemically small quantity of $H_2S^{35}O_4$ was added also.

Radiochemical Experiments. To examine possible identities among the products from Methods I and III, 20 mg. of unlabeled crude product of I was mixed with 2 mg. S^{35}-labeled[18] crude product of III. Aliquots of the mixture were fractionated by two-dimensional chromatography in solvents 1 and 2. Radioautographs of chromatograms were prepared, and the distribution of radioactive areas of chromatograms (resulting only from products of Method III) was compared visually with distribution of ultraviolet-absorbing areas of the same chromatograms[11] (main contribution from products of Method I). The visual observations of apparently coinciding localization of radioactive and ultraviolet-absorbing compounds were checked by appropriate instrumental measurements.[19]

For elementary analysis of sulfur, cytidine sulfates assumed to have equal S/S^{35} ratios were prepared by esterification of labeled H_2SO_4 with cytidine (Method III). The seven labeled products were separated on an analytical scale in duplicate. Products and appropriate blanks were eluted with water. To determine S^{35} activity in an eluate, a 0.0199 ml. aliquot and 0.5 ml. of water were transferred to a planchet, evaporated and counted. To determine moles of a cytidine sulfate in an eluate, an aliquot was acidified to *p*H 1.0 and analyzed spectrophotometrically as above. Molar radioactivity values were calculated as follows: $M.R. = \textit{radioactivity of eluate} \times \epsilon_{max} \times \textit{optical depth in cm./volume of eluate in l.} \times OD_{max}$, giving results as counts per min. S^{35} per mole of compound. Sulfur content was calculated from: $\textit{g. atoms S per mole} = \textit{M.R. of compound}/\textit{M.R. of CMS}$, where an average experimental M.R. value for CMS (3.985×10^{11}) was employed to best represent all the cytidine monosulfates.

Results

Proof of Structure of Cytidine 5′-Sulfate.—The following analytical results confirmed the chromatographic evidence[15] that the substance of the deposit designated 5′-CMS (Fig. 1) is cytidine 5′-sulfate: (a) on co-chromatography with the product of Method II in solvents 1 and 2, only 1 spot resulted; (b) elemental composition; (c) periodate oxidation[20] (0.99 moles per mole); (d) hydrolysis with strong acid yielded chromatographically identified cytidine (see Fig. 2) and sulfate. The possibility that the sulfate might be attached to the cytosine moiety as an N^4-sulfamino group was excluded on the bases of electrophoretic mobility properties (see Table III) and the fact that the sulfate group remained attached following deamination with nitrous acid.[21] The product of the deamination reaction was tentatively identified as uridine 5′-sulfate: it had R_m = 0.57 in solvent 1, an absorption spectrum like that of uridine, S^{35} radioactivity when prepared from 5′-CMS[35] and electrophoretic mobility equal to that of uridine 5′-phosphate at *p*H 3.4.

Identity of Products from Methods I and III.—That the products of Methods I and III are qualitatively identical was indicated by comparison of two-dimensional chromatograms of the respective crude products fractionated first dimension with solvent 1 and second dimension with solvents 2, 3 or 4. This was confirmed by the experiment wherein unlabeled crude product of I was co-chromatographed with radioactive crude product of III. Fig. 1 thus serves to identify the substances investigated for both methods.

(19) T. D. Price and P. B. Hudson, *Nucleonics*, **13**, 3, 54 (1955).

(20) G. Schmidt, R. Cubiles, N. Zollner, L. Hecht, N. Strickler, K. Seraidarian, M. Seraidarian and S. J. Thannhauser, *J. Biol. Chem.*, **192**, 715 (1951).

(21) J. Baddiley, G. W. Kenner, B. Lythgoe and A. R. Todd, *J. Chem. Soc.*, 657 (1944).

Sulfur Content.—Results of radiochemical analysis of products for sulfur content are presented in Table I.

TABLE I

RADIOCHEMICAL DETERMINATION OF SULFUR CONTENT

Compound	Molar radioactivity, c.p.m. mole^{-1} $\times 10^{-11}$	Gram atoms S mole^{-1}
Cytidine (2' and 3')-sulfates[a]	4.11	1.03
Cytidine 5'-sulfate	3.86	0.97
Cytidine 2',3'-disulfate	8.44	2.12
Cytidine 2',5'-disulfate	8.12	2.04
Cytidine 3',5'-disulfate	8.03	2.02
Cytidine 2',3',5'-trisulfate	12.65	3.18

[a] The 2'- and 3'- isomers were eluted and evaluated together after development 1 (*cf.* Fig. 1) in this study.

It is evident that the data establish with adequate accuracy the sulfation level of the compounds. Small departures from integer values for the computed S atoms per molecule may be ascribed to small variations of ϵ_{max} from the value 13,100 assumed in the calculations of molar radioactivity (see Experimental and Spectrophotometric Properties).

Composition of Product Mixtures.—Relative quantities of individual cytidine sulfates in synthetic mixtures from Methods I and III are summarized in Table II.

TABLE II

RELATIVE QUANTITIES OF CYTIDINE SULFATES IN THE SYNTHETIC MIXTURES

Compound	Mole % of total nucleoside sulfates Method I	Method III
Cytidine 2'-sulfate	2.04	2.95
Cytidine 3'-sulfate	2.89	4.81
Cytidine 5'-sulfate	59.4	51.4
Cytidine 2',3'-disulfate	..[a]	1.10
Cytidine 2',5'-disulfate	15.0	14.4
Cytidine 3',5'-disulfate	15.7	21.3
Cytidine 2',3',5'-trisulfate	4.38	4.02

[a] Detected fluoroscopically[11] but quantity in the chromatogram too small for evaluation.

Results obtained by chromatographic separation as in Fig. 1 and direct spectrophotometric analysis of eluates reveal that cytidine 5'-sulfate is the main product of both syntheses. Sulfation of cytidine proceeds maximally at its 5'-position and minimally at the 2'-position. Yields of total cytidine sulfates were 54 and 62% of the total cytidine used in the particular syntheses detailed for Methods I and III respectively. When Method III was applied to only 1.57 μmole of cytidine, a lower yield of 8.5% resulted: such yield is nevertheless useful in preparation of S^{35}-labeled cytidine sulfates of very high specific radioactivity from inexpensive reactants.

Electrophoretic Mobilities.—Because it remained possible that some of the sulfur atoms (Table I) might be involved in sulfamino, pyrosulfate or cyclic sulfate groups, direct evidence that served to exclude these possibilities was obtained by the paper electrophoretic studies summarized in Table III.

It will be observed that 2'-CMS and 3'-CMS and 5'-CMS all have mobilities equal to that of 5'-CMP at pH 3.4, as would be predicted for compounds of equal charge and mass. If one of the cytidine monosulfates had contained a sulfamino group at carbon 4 of the cytosine moiety, it would have lacked a partial positive charge (pK_a of primary amino group of cytidine = 4.1)[22] and exhibited markedly greater mobility toward the anode. A cyclic monosulfate, if present, would have lacked a negative charge and moved toward the cathode instead of the anode. The 3 cytidine disulfates moved together with mobility more than twice that of cytidine monosulfates. The observed increasing electrophoretic mobility with increasing degree of sulfation would not have occurred if, in any instance, the increased sulfation level had been a manifestation of presence in the product mixture of a hypothetical nucleoside pyrosulfate. At *p*H 9.0 in borate buffer, the 5'-CMS had a mobility similar to that of the cytidine disulfates, showing availability in 5'-CMS of unsubstituted 2'- and 3'- *cis*-hydroxyl groups capable of reacting with borate to form a negatively charged complex.[23]

TABLE III

ELECTROPHORETIC MOBILITIES OF CYTIDINE SULFATES

Fraction	Mobility relative to 5'-CMP *p*H 3.4[a]	*p*H 9.0[b]
Cytidine (2' and 3')-sulfates	1.0	0.64
Cytidine 5'-sulfate	1.0	.91
Mixed cytidine disulfates	2.9	.89
Cytidine 2',3',5'-trisulfate	4.3	1.2

[a] 0.02 *M* formate buffer, 1,000 volts, 45 min. [b] 0.10 *M* borate buffer, 1,000 volts, 25 min.

Chromatographic Mobilities.—Paper chromatographic solvents originally developed for separations of nucleotides appear to be generally applicable to nucleoside sulfates. Table IV shows behavior of these compounds in four of the widely used solvents.

TABLE IV

CHROMATOGRAPHIC MOBILITIES OF CYTIDINE SULFATES

Compound	Mobility relative to 5'-CMP[a] Solvent 1	Solvent 2	Solvent 3	Solvent 4
2'-CMS	0.98	0.84	3.25	0.92
3'-CMS	.95	.68	3.25	.92
5'-CMS	.82	.63	3.02	.77
2',3'-CDS	.59	.80	2.54	.27
2',5'-CDS	.50	.70	2.54	.21
3',5'-CDS	.50	.57	2.54	.21
2',3',5'-CTS	.32	.67	1.98	.08

[a] Test solvents were employed in + X direction (*cf.* Fig. 1, across grain of paper) after a preliminary development + Y in solvent 1, thorough drying, and application of the 5'-CMP reference standard. Development times for solvents 1, 2, 3 and 4 were 28, 18, 18 and 19 hr. respectively, descending flow.

Uses of solvents 1 and 2 have been noted above. It may be added that cytidine and sulfate have relative mobilities of 2.0 and 0.49 in solvent 1, and pyridine moves near the solvent front. In solvent 2, sulfate moves at the solvent front. In the ammoniacal propanol solvent 3 all of the cytidine sulfates are characterized by mobilities higher than that of cytidine phosphate, a property of potential value in biochemical experiments. An-

(22) J. J. Fox, L. F. Cavalieri and N. Chang, *J. Am. Chem. Soc.*, **75**, 4315 (1953).

(23) J. X. Khym and L. P. Zill, *ibid.*, **74**, 2090 (1952).

other feature of interest emerged with this relatively non-polar, alkaline solvent when applied to cytidine sulfate samples that had been saturated with H_3BO_3 before application to the chromatogram. Presence of the borate decreased mobility of 5′-CMS by a factor of 0.53 without affecting mobilities of any of the other cytidine sulfates: this clear inability of the other cytidine sulfates to form borate complexes[23] established that they are all sulfated at 2′- and/or 3′-positions. Solvent 4 moves cytidine sulfates having a single negative charge much faster than others having multiple charges, a generality observed before in application of the solvent to nucleoside phosphates.[11]

Spectrophotometric Properties.—Ultraviolet absorption spectra of all seven cytidine sulfates resemble rather closely the spectra of cytidine[24] at investigated *p*H values of 1 and 7. Molecular extinction coefficients of 5′-CMS were determined for these *p*H values and found to be within 4% of those of 5′-CMP at all wave lengths in the 210 to 290 mμ range. Among the different cytidine monosulfates, small but significant differences in wave lengths of absorption maxima could be detected, as demonstrated in Table V. These correspond closely to the differences among corresponding isomeric cytidine monophosphates (data of W. E. Cohn[25]). Wave lengths of absorption maxima and minima of cytidine monosulfates were determined additionally at *p*H values of 13 and 14, to obtain still further evidence useful in distinguishing the 2′-CMS from the 3′-CMS (Fig. 1). Significant increases of λ_{max} and λ_{min} were found (Table V) for 3′-CMS, but not for 2′-CMS, on increasing the *p*H from 13 to 14: a concomitant 6% increase of OD_{274} occurred for 3′-CMS. These increases are the expected manifestations of the presence in cytidine 3′-sulfate of an unsubstituted, ionizable hydroxyl group at the 2′-position.[24]

TABLE V

SPECTROPHOTOMETRIC CONSTANTS OF CYTIDINE MONOSULFATES

Compound	*p*H	λ_{max}	λ_{min}	OD_{250}/OD_{260}	OD_{280}/OD_{260}
2′-CMS	1	277	239	0.56	1.62
	7	268	250	.93	0.76
	13	268	250	..	..
	14	269	250	..	..
3′-CMS	1	278	240	.48	1.98
	7	269	250	.90	0.88
	13	269	250	..	..
	14	273	253	..	..
5′-CMS	1	280	240	.48	2.07
	7	271	250	.88	0.95
	13	271	250	..	..
	14	274	252	..	..

Detailed photometric studies have been done on the cytidine di- and tri-sulfates only at *p*H 1. The equation used to compute the radiochemical data of Table I (see Experimental) was solved for ϵ_{max} as a means to determine this photometric constant from more detailed consideration of the same data: ϵ_{max} = (*M.R.* × OD_{max} × *volume of eluate in l.*)/(*radioactivity of eluate* × *optical depth in cm.*). The equation can be used if an *exact* value of M.R. is available. Owing to prior limited knowledge of *exact* ϵ_{max} values, the only compound for which the M.R. could be considered strictly quantitative in Table I was 5′-CMS. This value of 3.86×10^{11} will suffice, however, for it represents the c.p.m. per g.-equivalent of sulfur, whence *exact* M.R. values × 10^{-11} of all cytidine mono-, di- and trisulfates must be 3.86, $2 \times 3.86 = 7.72$ and $3 \times 3.86 = 11.58$ respectively. Substituting these M.R. values in the equation, the following values of ϵ_{max} were obtained: (2′&3′)-CMS, 12,300; 2′,3′-CDS, 11,980; 2′,5′-CDS, 12,440; 3′,5′-CDS, 12,600; 2′,3′,5′-CTS, 12,020. The properties again have a striking similarity to those of corresponding cytidine phosphates, where the 2′- and 3′-monophsphate isomers have ϵ_{max} values at *p*H 1 about 5% lower than the 5′-isomer,[25] and ϵ_{max} of deoxycytidine 3′,5′-diphosphate is about 3% lower.[26]

Stability of Cytidine Sulfates.—Stability of 5′-CMS to hydrolysis by strong acid was investigated by heating with 2 *N* hydrochloric acid at 100° in sealed glass tubes. Samples withdrawn periodically were chromatographed in solvent 1 and the separated 5′-CMS and cytidine analyzed spectrophotometrically. Inspection of results plotted in Fig. 2 reveals that 5′-CMS is much more acid-labile

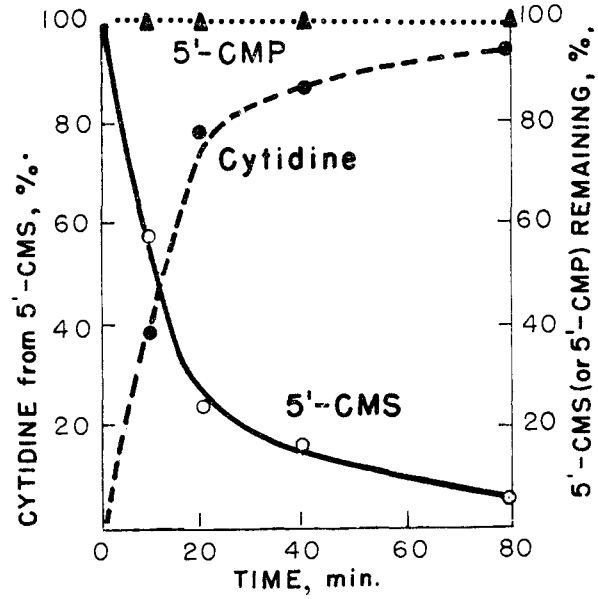

Fig. 2.—Kinetics of acid hydrolysis of cytidine 5′-sulfate and cytidine 5′-phosphate.

than 5′-CMP. Hydrolysis of the primary sulfate ester bond was nearly complete by 80 min., and cytidine was the only ultraviolet-absorbing hydrolysis product present in appreciable quantity at any time. In water at 0°, 5′-CMS was found to be stable for 1 month. Stability of 5′-CMS in alkali was investigated by incubating in 1.0 *N*

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lithium hydroxide for 18 hr. at 37°. This treatment resulted in 5.6% deamination as indicated by appearance in the chromatograms of the previously indicated compound obtainable by treatment with nitrous acid, presumably uridine 5′-sulfate. No cytidine, uridine, sulfate or other degradation products could be detected, showing stability of the sulfate ester link under these alkaline conditions.

The only systematic study conducted on stability of all the cytidine sulfates was a test with the very mild acid hydrolysis of Tsuboi,[27] which is designed to degrade pyrophosphate bonds but not phosphomonoester bonds. None of the cytidine sulfates were appreciably hydrolyzed by this procedure, indicating that at least moderate acid stability characterizes both primary and secondary sulfate ester bonds with the ribose of cytidine.

Discussion

Although the six synthetic products other than 5′-CMS were not isolated in crystalline form, their identities have been established with reasonable certainty. Spectrochemical properties distinguish 2′-CMS from 3′-CMS, as does behavior in chromatographic solvent 2 (Fig. 1), which moves nucleoside 2′-phosphates faster than nucleoside 3′-phosphates.[8,15] 2′,3′,5′-CTS is the only theoretical cytidine trisulfate that would have the observed high electrophoretic mobilities (Table III) unless sulfation of the 4-amino group of the cytosine occurred. That this product could really include a sulfamino group is very unlikely because of the typical cytidine absorption curve, its at least moderate stability to acid, and the fact that all the cytidine monosulfates clearly have their sulfate moiety attached at a 2′-, 3′- or 5′-position and not at the N^4-position.

Identification of the isomeric status of the individual cytidine disulfates is supported by less rigorous evidence. Since 2′-CMS and 3′-CMS have significantly higher mobilities than 5′-CMS in solvents 1, 2 and 4, one basis for identification of 2′,3′-CDS is as the fastest-moving cytidine disulfate in these solvents (Fig. 1, Table IV). Further evidence is that the designation 2′,3′-CDS is assigned to the cytidine disulfate synthesized in lowest yield (Table II), which would be expected for this isomer because (i) rather low yields of 2′-CMS and 3′-CMS show that sulfation efficiency at 2′- and 3′-positions of the ribose is relatively unfavored, and (ii) disulfation at the proximal positions may be impeded by steric hindrance. Distinction between 2′,5′-CDS and 3′,5′-CDS rests only upon comparisons of relative synthetic yields and chromatographic mobilities with those of related compounds: (i) yields of 2′,5′-CDS are a little lower than those of 3′,5′-CDS in the same way that yields of 2′-CMS are lower than those of 3′-CMS (Table II), (ii) in solvent 2, a sulfate at 2′-position confers higher mobility than a 3′-sulfate in the monosulfate series, and would be expected to do so in the disulfate series (Fig. 1), and (iii) although analogous cytidine diphosphates could not be separated in solvent 2[15], a clear resolution by this solvent was obtainable for comparable adenosine diphosphates; adenosine 2′,5′-diphosphate had a higher mobility (R_m = 0.47) than adenosine 3′,5′-diphosphate (R_m = 0.39). Our limited spectrophotometric data on the cytidine disulfates support their isomeric allocations within the framework of a theory that sulfate (or phosphate[25,26]) esterification at the 3′-position of cytidine causes a small hypochromic shift in the acid absorption curve, and esterification at the 2′-position increases the extent of the shift. The available evidence supports this theory.

A unifying result of the present study is the separation and recognition of all seven of the possible pentose-sulfated derivatives of cytidine. In this connection, it may be noted that two of the compounds, 2′,3′-CDS and 2′,3′,5′-CTS, apparently do not have reported counterparts in any nucleoside phosphate series. However, 2′,3′,5′-trimethylsulfonyloxyuridine has been synthesized.[28]

Considering the method of synthesis and separation employed by Egami and Takahashi for adenosine sulfates,[2] it appears possible that their fractions were comprised of components with isomeric composition as found here for the cytidine sulfates.

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